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Effect of plant growth promoting rhizobacteria on early growth of Rice plant (*Oryza sativa* L.) under Cadmium (Cd) and Lead (Pb) stress condition.

Amit Kumar Pal and Chandan Sengupta
Microbiology Research Laboratory, Department of Botany, University of Kalyani, Kalyani – 741 235, West Bengal, India.
chandansenguptaku@gmail.com

ABSTRACT:
Microbes assisted phyto-remediation technique are widely applied for reducing the risk of heavy metals contamination in the soil. The present study was designed to observe the effect of rhizobacteria on plant growth enhancement under heavy metal stress condition. We examined the growth of rice plant with two isolated PGPR strains under different test concentration (ppm) of Cd and/or Pb (i.e. 20,40and 60ppm). In all studied cases, we observed that different growth parameters in the un inoculated heavy metal treated rice plants (i.e-without PGPR) were reduced as compared with control plants. Cd and Pb toxicity were assessed by observing different growth parameters like stunted growth and poor rooting and low chlorophyll content. Inoculation of PGPR in rice plant reduced the deleterious effects of heavy metals to some extent and enhanced the different growth parameters as compared with un inoculated heavy metal treated plants. Our present study conducted with 2out of 20 different bacterial isolates but observed results indicated that KUBM 20 strain of enhanced more in the growth of rice plant under heavy metal stress condition than KUBM 1.

Key words: Cadmium stress, Lead stress, PGPR, *Oryza sativa* L. Early vegetative growth, sustainable agriculture.

INTRODUCTION

In recent time contamination of agricultural field with heavy metals is a burning problem for development of sustainable agriculture which eventually affected the food security and human health [21] . Among the various causes of soil contamination with heavy metals atmospheric deposition or disposal of sewage lead to leaching of metals are prime important [2]. These leachy metals may contaminate ground water which subsequently accumulated in soils [11]. The range of metal contamination in soil is variable and it mainly depends on human activity or geological origin of soil [4]. Such contamination have profound influence on natural ecosystem of mainly terrestrial and water body [15]. Except few, most of the heavy metals are mobile in nature and therefore move through soil pore and finally uptaken by plant roots. Among these heavy metals available in the soil some are such as Fe, Zn, Mg and Ca are essential micronutrients for plant body and thus need in small quantity, but if their concentrations are high in plant body then it will not good and caused toxic effect [14]. Zinc is the masculine element which is essential for reproductive activity, balances the level of copper in body [17] and acts as a co-factor for dehydrogenating enzymes [9]. Among the others, Cd and Pb have no direct beneficial effects on plants and animals. In contrast, in excess amount, they caused toxic effects [2,7] . Cd can be contaminated in the environment through anthropogenic sources, paints, batteries, coating on metal devices plastic stabilizer [5,19], excessive use of phosphate fertilizer containing Cd from rock phosphate in agricultural land [1] etc. For direct or indirect toxic effects of heavy metals on life system, the removal of these metals from water and soil are prerequisite but the clean up procedure of heavy metals are expensive, destructive and time consuming [15]. Scientist are engaged themselves to develop some cost effective devices of which exploitation of some specific microorganism are the prime important [27]. Many researchers have noticed that, some plant species are endemic to metalliferous soils and able to withstand in presence of heavy metals in soils [4]. Simultaneously it has been noted that the influence of plant growth promoting rhizobacteria (PGPR) have great role to nullified the adverse effects of heavy metal deleterious effects on plant growth and development, as these PGPR can directly accelerate the phytoremediation process by altering the bioavailability of metals via changes in production of phytohormones, siderophores, varying pH, moreover increased release of metal binding chelators [13]. In this study the preliminary objective is that to estimate the adverse effects of Cd and Pb on early seedling growth of rice plants. In addition to that the whole plant biomass and chlorophyll content of the young plant was also measured . In a separate study ascertained how the adverse effects of heavy metals were overcome by use of two Cd and Pb tolerant PGPR strains.

MATERIALS AND METHODS:

Collection of soil samples-

Soil samples were collected from the rhizosphere of the agricultural field in Nadia district,West Bengal,India, which are frequently irrigated with industrial effluent water. The rhizospheric soils were collected in plastic bags and stored in aseptic

condition in clean dry places and used within 7 days of collection.

Collection of rice seeds-
Rice seeds were collected from the Central rice research institution, Chinsurah, west Bengal, India (variety miniket) brought to the laboratory and stored in cooled, dust free condition.

Isolation of bacteria from soil-
Bacterial strains from the collected soil samples were isolated following soil dilution plate count agar technique using NA (Peptone – 5.0 ,Beef extract – 3.0 , Agar – 15.0 , Nacl – 5.0 , pH -7.0 , Water – 1 liter) medium. Plates with soil inoculants were aseptically incubated in incubator (37^0C) and the bacteria colonies thus, developed after 48 hrs, were separately collected on agar slopes in tubes.

Detection of minimal inhibitory concentration (MIC) of Cd and Pb of the isolated strains-
The cadmium(Cd) and Lead(Pb) tolerance level of the isolated bacterial strains were detected by streaking of each bacterial strains on NA medium containing different concentration (50,100,150…..ppm) of Cd or Pb. Higher concentrations of Cd or Pb tolerant bacterial strains were selected for further studies.

Preliminary characterization of isolated bacterial strains-
The prominent individual bacterial isolates were characterized by their colony morphology, staining properties and biochemical tests. The shape, margin, elevation and colour of the bacterial colonies were recorded. Biochemical tests such as Catalase, Amylase, Gelatine hydrolysis tests were performed with each individual bacterial isolates.

Determination of plant growth promoting ability-

Determination of ammonia production-
Selected bacterial isolates were inoculated separately in peptone water medium (Peptone 10 g, NaCl 5 g, Distilled water 1lit,PH-7) and incubated at 37^0c for 4 days. 1ml of Nesseler's reagent was added in each tubes and development of faint yellow to brown colour are observed for low, moderate to high ammonia production respectively.

Determination of phosphate solubilization-
Each selected bacterial strains were streaked on Pikovskaya's agar (Himedia) medium separately and incubated at 37^0c for 3 days. Hallow zone were observed around the streak for positive result of phosphate sollubilization.

Determination of Indol acetic acid production (IAA)-
Each bacterial strains were inoculated to 20 ml of Luria Bertani (LB) (Tryptone 10g, Yeast extract 5g, NaCl 10 g, Agar 20g Distilled water 1 lit.,PH 7) supplemented with L-tryptophan (0.2%) and incubated in 24^oc rotary shaker then centrifuged at 10000g for 15 min. 2 ml of supernatant was collected and 2-3 drops of O-phosphoric acid along with 4 ml of salkowski's reagent added (100 ml of 35% of perchloric acid along with 2 ml of freshly prepared 0.5 M $FeCl_3$ solution). Then incubated for 30 min in dark room. Absorbance were recorded in 530 nm. Quantity of auxin were calculated from the standard curve using indol acetic acid as standard (10-100µg).

Detection of seed germination percentage-
The rice seeds were surface sterilized by 0.1% $HgCl_2$ for 3 minutes followed by several wash with sterilized distilled water. After 24 hrs. the seeds were placed on the sterile blotting paper with 3 different concentrations (20ppm,40ppm and 60 ppm) of Cd or Pb and combination of heavy metals (Cd and Pb in 1:1 ratio) on Petri plates . In control sets the seeds were treated only with distilled water . Number of seed germination were recorded after 24 hrs. intervals upto 6 days.

Germination percentage= (Total no of germinated seeds /Total no of seeds)*100

Results and discussion

Isolation and characterization of bacteria-
The bacterial strains were isolated from the soil sample by standard dilution plate count technique as described in material and methods. Among the 20 isolated bacteria, their Cd and Pb tolerance level were checked and noted that the ability to withstand high concentration of Cd and Pb in soil ecosystem.

Table 1: Colony morphology of bacterial isolates , Gram nature, MIC of Cadmium (Cd) and Lead (Pb),Biochemical tests and PGPR confirmation tests

Name of strains	Colony morphology	Gram nature	Cd tolerance(MIC)(ppm)	Pb tolerance(MIC)(ppm)	Amylase	Catalase	Gelatin hydrolysis	Phosphate solubilizition	NH_3 production	IAA production
KUBM 1	Creamish white, undulate	+ ve	200	500	+	-	++	+	++	-
KUBM 20	White, filamentous	+ve	200	400	++	++	++	+++	++	++

('+' or '-' sign indicate the positive or negative approach of the test. No of '+' sign denote the intensity of yellow colouration, bubble production or liquefaction of gelatin respectively for amylase, catalase and gelatin hydrolysis test.)

Different biochemical tests has been observed (table no 1). In a separate study both the bacteria were tested in their various plant growth promoting activity and the results are depicted in table no 1. It was noted that as compared to KUBM 1, KUBM 20 showed better plant growth promoting activity as it is a better IAA producer, Phosphate solubilizer as well as ammonia producer .

Exploitation of PGPR bacteria on growth of rice seedlings in heavy metal stress condition-

The germinated seeds were placed in culture tubes (size-25mm *150mm) supplemented with 25 ml of minimal nutritional support as MS medium with solutions of different heavy metals stress with two selected bacteria alternately . Seeds inoculated in MS medium without any heavy metals and bacteria are considered as control set. The experiment was continued for a period of 14 days in exposed sunlight condition in the laboratory . These tubes were randomized each day in the green house to eliminate the position effects.

At the end of 14 days of seedling growth plants were harvested followed by root portions were blot dried and kept for the future study. For estimation of growth of rice seedlings, we mainly observed some major growth parameter such as – 1)root length, 2) shoot length, 3) dry weight, 4) fresh weight and 5) chlorophyll content by using spectrophotometer (Microprocessor visible Spectrophotometer, model no-LI-722, Lasany) .

Effects of PGPR on seed germination under heavy metal stress condition

Effects of different heavy metals in combination with test PGPR strains used in this study are depicted in the figure 1. The results showed that the germination percentage was severely affected in presence of heavy metals. Among the two metals used, germination was seriously affected by using the concentration of 20 ppm Cd as compared to control set containing no Cd or Pb. The advance effect of Cd by use of 20 ppm was previously reported by Aydinalp and Marinova(3) (2009) in their study on alfa alfa seedling . The results obtained in this study thus in confirmation of the previous report. Moreover when the concentration increased then the percentage of rice seed germination was also decreased as compared to control sets and it was 14.4,91.4 and 94.8 % under 20,40 and 60 ppm of Cd respectively, as result presented in figure 1 . The results further showed that in presence of PGPR strains, the adverse effect of Cd on seed germination was improved by 0.13,0.20 and 0.82 fold by inoculation of KUBM 1, moreover for KUBM 20 inoculation, germination was improved by 0.11, 0.32 and 1.07 fold under test different concentration of Cd respectively as compared with uninoculated treated plants. Although the percentage of retrieve the germination is variable . It was cleared from the observation that S55 strain is more effective and showed maximum improvement. The results therefore confirmed the observations made by many worker (23,3,10,25) in which they reported that Cd stress decreased seed germination of different crops.

Different biochemical tests has been observed (table no 1). In a separate study both the bacteria were tested in their various plant growth promoting activity and the results are depicted in table no 1. It was noted that as compared to KUBM 1, KUBM 20 showed better plant growth promoting activity as it is a better IAA producer, Phosphate solubilizer as well as ammonia producer .

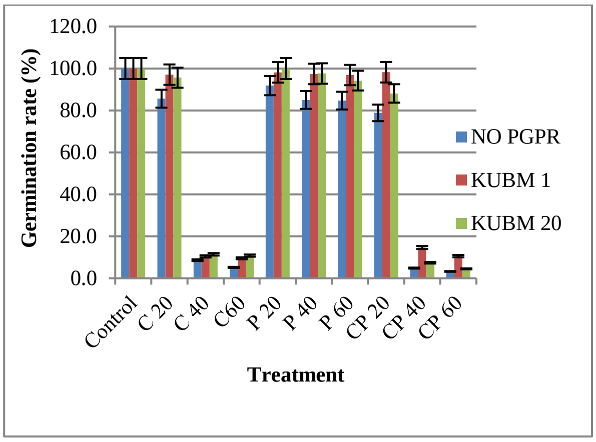

Fig. 1. Seed germination after two weeks of exposure to heavy metals with PGPRs.

Similar results were observed when Pb was used as heavy metal. Here also, increase of metal concentration , decreased the germination rate of seeds linearly and PGPR strains KUBM 20 showed maximum beneficial effects as compared to KUBM 1 irrespective of heavy metal percentage were used.

In a separate experiment the combination of two test heavy metals on germination was studied. The results showed (figure-1) that here also the percentage of germination was reduced and it was much more than any of the test metal did alone. This reduction were by 8.1,15 and 15.3% respectively for different cumulative heavy metal concentration as compared with control plants . Further more it was also noted that PGPR strains nullified the adverse effects and increase the percentage of germination to some extent strain KUBM 1 which enhanced the germination percentage by 0.24,1.98 and 2.28 fold as compared with uninoculated treatment plants respectively but KUBM 20 strain enhanced 0.11,0.51 and 0.41 fold.

It was of interest to note that PGPR strains had some positive effects on seed germination and reduce the adverse effects of heavy metals in contaminated soils. The results thus obtained in this study clearly indicated that PGPR may safely used for enhancement of seed germination in heavy metal contaminated soil.

Effects of PGPR on root length (cm) under heavy metal stress condition-

The results obtained in relation to root growth is depicted in figure no 2, which indicated that with gradual increase the Cd concentration in the medium from 20 to 60 ppm the root length was decreased simultaneously.

The results further showed that the percentage of decrease were 42.6 ,55 and 74.55% respectively as compared to control plants under 20,40 and 60ppm cd respectively. In a subsequent experiment when isolated plant growth promoting rhizobacterial strains KUBM 20 was inoculated in the medium then the adverse effect of Cd was partly nullified and it enhanced the root length by 0.77,1.16,2.7 fold but in case of KUBM 1 inoculation the root length enhancement were by 0.34,0.55,1.30 fold as compared with uninoculated treatment under 20,40 and 60ppm cd stress respectively.

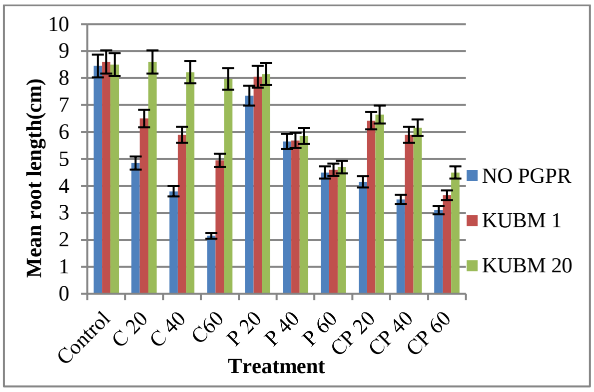

Fig. 2. Root length after two weeks of exposure to heavy metal enriched medium inoculating with PGPRs

In a separate study when Pb was applied as heavy metal, then the trend of root length reduction was same. Here also due to increase the Pb concentration simultaneously decrease the root length and application of bacterial strain KUBM 20 reduced the adverse effect of Pb in a considerable percentage (20,40 and 60 ppm). The experiment was further extended in which Cd and Pb was used in combined (1:1 ratio) and the result showed that the combined adverse effect of both the metals was cumulative. Here also the percentage of root length was reduced as compared to control i.e. heavy metal free condition. Aydinalp and Marinova (3) observed that under increasing Cd stress root length and shoot were gradually decreased. The possible explanation for this increase in the KUBM 20 inoculated plants under heavy metal stress condition, may be due to production of indol acetic acid (IAA) . Rajkumar and Freitas (22) also observed that *Pseudomonas* and *Arthrobacter* produce IAA that produce better growth and development.

Effects of PGPR on shoot length (cm) under heavy metal stress condition-

The effect of Cd and Pb on rice shoot growth was examined and the results presented in Figure 3. It was indicated from the results that shoot growth was also severely affected like root growth in presence of heavy metal stress condition. Here also 26.54,32.3 and 38.9 % of shoot growth was reduced due to soil contamination with Cd concentrations respectively, on the other hand 21.2,24.3 and 30.5% of shoot growth reduction observed under 20,40 and 60 ppm of Pb treatment. After inoculating of PGPR, it certainly reduce the effect of Cd and Pb stress and enhance the shoot growth to some extent than the uninoculated treated plants.

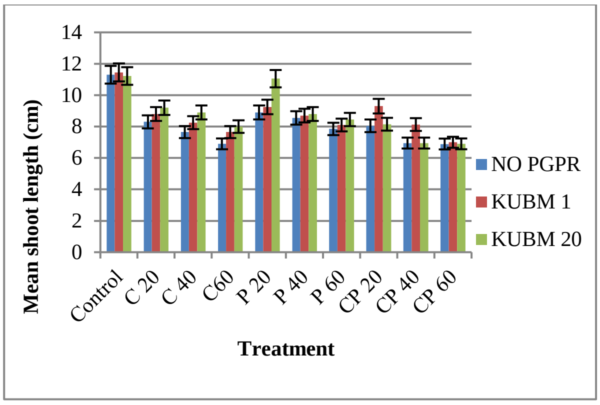

Fig. 3. shoot length after two weeks of exposure to heavy metal enriched medium inoculating with PGPRs

Another important findings obtained from this results, that adverse effect was gradually increased with gradual increase in heavy metal concentrations as noted previously by Aydinalp and Marinova (3).They observed simillar kind of result where 20 ppm of Cd reduced the shoot growth by 62% in case of their study on alfaalfa plants. The results further indicated that at low concentration of heavy metal/s may have positive effects on growth of the rice plants. Rajkumar and Freitas (22) observed that the plant growth was promoted PGPR inoculation and the root and shoot length of PGPR inoculated plants improved by 30-37% as compared to uninoculated plant in the heavy metal stress condition.

Effects of PGPR on fresh weight (mg/seed ling) under heavy metal stress condition-

The effect of fresh weight on rice plant due to presence of heavy metals in soil in combination with PGPR strains was estimated. The result presented in figure 4 . It is noted that presence of Cd or Pb, the fresh weight of rice plant was significantly affected and reduce in several fold as compare to control set without any heavy metal.

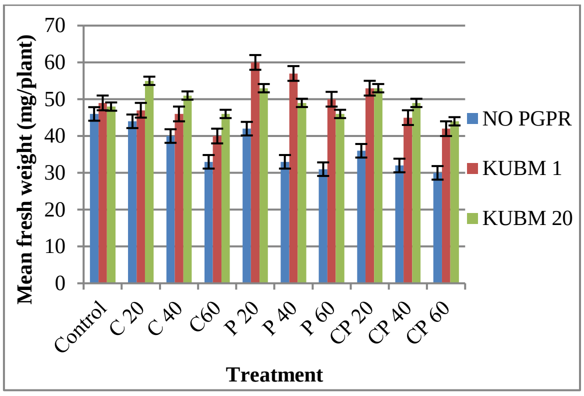

Fig. 4. Fresh weight after two weeks of exposure to heavy metal enriched medium inoculating with PGPRs

The results further indicated that the effect of Cd is much more than Pb. The data obtained in this study therefore in confirmation of the previous findings. In this study reduction in mean fresh weight were 4,13 , 28 % under Cd stress, 8,28 ,32% under Pb stress and 21,30,34% under cumulative Cd and Pb stress(1:1 ratio) respectively for 20,40 amd 60 ppm treatment. The result further showed that in presence of PGPR strains in Cd and Pb inoculated soils the adverse effects was partially nullified. Under Cd stress, KUBM 1 strain enhanced in mean fresh weight by 0.06,0.15,0.21 fold and KUBM 20 strain enhanced 0.19,0.10,0.39 fold respectively for 20,40 and 60 ppm. Similar kind of results were noted in case of PGPR inoculated Pb stress and cumulative Cd +Pb stress where PGPR inoculation reduced the effects of heavy metals and enhanced the shoot weight. More over among the PGPR strains KUBM 20 strains was more potential than other to overcome the deleterious effect of soil contamination with heavy metals. Similar type of observation was made recently by Kamran et al (12) in case of *Eruca sativa* where inoculation of bacteria caused overcome the adverse effect of heavy metal. Plant biomass increasing is due due to production of different phytohormones like Gibberellins, IAA etc (22). The observation in this

study therefore support the previous views as mentioned above.

Effects of PGPR on dry weight under heavy metal stress condition-

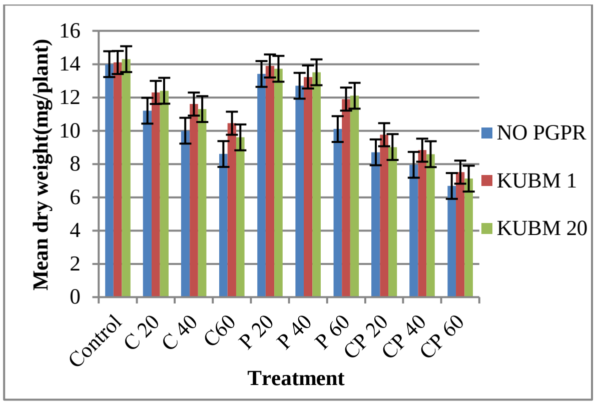

Fig. 5. Dry weight after two weeks of exposure to heavy metal enriched medium inoculating with PGPRs

In a separate study the effect of heavy metals on dry weight of rice plant was observed depicted in the figure 5. The plant were allowed in the heavy metal Cd or Pb incorporated soil as compared to control set. After a couple of weeks of growth, the plants were uprooted and oven dried at 80^0 c for 24 hr. The results showed that the dry weight of plant grown in heavy metal contaminated soil was reduced . Like the previous study in this report here also confirm that heavy metals had some deleterious effects on dry weight of plants. It can be concluded that the effects of Cd were more severe than the effect of Pb in case of the dry weight production. Moreover the study further showed that application of PGPR strains overcome the heavy metal 's adverse effects to some extend although the degree of recovery was variable based on mainly the inoculated bacterial strains and the nature of heavy metals. Our study is similar to the findings of Kamran et al.(12), that the PGPR increases dry weight under heavy metal stress condition.

Effects of PGPR on total chlorophyll content under heavy metal stress condition-

Finally the effect of heavy metals on total chlorophyll content on the treated plant ascertained. It was observed that heavy metal contamination resulted the deletion of total chlorophyll content as compared to control set (figure 6) .Here also found that heavy metal not only reduced the shoot length but also reduced the content of chlorophyll in plants. Under Cd stress condition severe loss in chlorophyll content has been observed beyond 20 ppm concentration and this reduction was by 24.96,53.24 and 67.96 % as compared with the control set respectively for 20,40,60 ppm of Cd. These data corresponded with the study of Oncel et al. (18). They observed that Cd reduces the chlorophyll content in wheat. According to Somashekaraiah et al.(24), Cd inhibit the chlorophyll biosynthesis and reduces the amount of chlorophyll in green plants. According to Patsikka et al.(20) leads to the degradation of chlorophyll pigments.

In another experiment inoculation of KUBM 20 surely reduced the effect of Cd on chlorophyll biosynthesis. KUBM 20 enhanced 0.26,0.96 and 1.5 fold as compared respectively with 20,40 and 60 ppm Cd treated plants. Moreover in the further experiments it can be concluded that Cd is the more deleterious than Pb. In cumulative effect of Cd and Pb shows more reduction in chlorophyll content than any heavy metal alone, but there also KUBM 20 reduced the effects of heavy metals in biosynthesis of chlorophyll content to some extent. Zhang et al. (29) also reported that rhizobacteria positively increased the chlorophyll content under Cd stress condition.

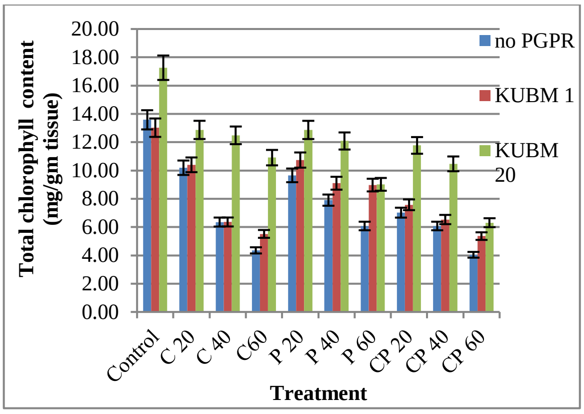

Fig. 6. Chlorophyll content (mg/ gm tissue) after two weeks of exposure to heavy metal enriched medium inoculating with PGPRs

CONCLUSSIONS

The results obtained through out the study , it suggested that contamination of soil by heavy metals is unhealthy for plant growth and development and it eventually costly for the agricultural development. Moreover the adverse effects of heavy metals may be partially or fully reduced by application of suitable PGPR strains. For this searching of proper bacterial strains and its application time and procedure need to be develop on cost intensive basis. Although few reports

(6,8,26) are available in relation to this but more works in this regard are necessary for development of sustainable agriculture. Furthermore, the knowledge about isolation of suitable PGPR strains, their application, proper dose maintenance and determine the inoculum are necessary for development and eventually upliftment of any inoculation program in heavy metal contaminated soil environment.

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